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Mitochondrial function and reactive oxygen species action in relation to boar motility☆

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Abstract

Flow cytometric assays of viable boar sperm were developed to measure reactive oxygen species (ROS) formation (oxidization of hydroethidine to ethidium), membrane lipid peroxidation (oxidation of lipophilic probe C_{11}-BODIPY$^{581/591}$), and mitochondrial inner transmembrane potential ($\Delta\Psi_m$; aggregation of mitochondrial probe JC-1) during hypothermic liquid storage and freeze-thawing of boar semen and to investigate relationships among ROS, motility, $\Delta\Psi_m$, and ATP production. Basal ROS formation and membrane lipid peroxidation were low in viable sperm of both fresh and frozen-thawed semen, affecting $\leq$4%. Sperm in fresh, liquid-stored and frozen-thawed semen appeared to be equally susceptible to the activity ROS generators xanthine/xanthine oxidase, $FeSO_4$/ascorbate, and hydrogen peroxide (H_2O_2). Of the ROS generators tested, $FeSO_4$/ascorbate was specific for membrane lipid peroxidation, whereas menadione, xanthine/xanthine oxidase, and H_2O_2 were specific for oxidization of hydroethidine. Menadione (30 μM) and H_2O_2 (300 μM) decreased ($P < 0.05$) motility by 90% during 60 min of incubation. Menadione decreased ($P < 0.05$) the incidence of sperm with high $\Delta\Psi_m$ by 95% during 60 min of the incubation, although ATP content was not decreased ($P > 0.05$) until 120 min. In contrast, H_2O_2 did not affect $\Delta\Psi_m$ or ATP at any time. The formation of ROS was not associated with any change in viability (90%) for either menadione or H_2O_2 through 120 min. Overall, the inhibitory affects of ROS on motility point to a mitochondrial-independent mechanism. The reduction in motility may have been due to an ROS-induced lesion in ATP utilization or in the contractile apparatus of the flagellum.
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1. Introduction

The fertility of boar sperm after freeze-thawing or long-term hypothermic liquid storage is less than that of fresh liquid semen [1,2]. It has been suggested that part of this reduction in sperm fertility may be due to oxidative damage from inappropriate formation of reactive oxygen species (ROS), or from subsequent membrane lipid peroxidation [3–6].

Mitochondria are the major site of intracellular ROS formation which results in a disruption of electron transport [7]. The coupling of electron transport to oxidative phosphorylation maintains a high mitochondrial inner transmembrane potential ($\Delta\Psi_m$) required for mitochondrial ATP production in somatic cells [8]. Therefore, one action of ROS in sperm could be the uncoupling of electron transport

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and oxidative phosphorylation, with a coincident reduction in the number of sperm containing a $\Delta\Psi_m$ high enough to support mitochondrial ATP production and sperm motility. It is likely that hydrogen peroxide (H_2O_2) is responsible for much of the damage to cell structure and function because of its high membrane permeability and the abundance of mitochondrial and cytoplasmic superoxide dismutase in mammalian sperm [9]. In addition to disruption of mitochondrial energy production, the production of the hydroxyl radical from H_2O_2 and existing lipid hydroperoxides through the Fenton reaction and from superoxide ($^{\bullet}O_2$) through the Haber–Weiss reaction, are major threats to peroxidation of membrane lipids [7,10–12].

2. Materials and methods

2.1. ROS formation

Typically $^{\bullet}O_2$ and H_2O_2 in spermatozoa have been measured by chemiluminescence procedures using lucigenin and luminol. However, these assays are problematic because of their poor specificity [13]. As an alternative approach, we developed a flow cytometric procedure, based on the ROS-induced oxidation of hydroethidine (HE) to ethidium, combined with the impermeant nuclear dye Yo Pro-1 to electronically gate out nonviable cells [14]. Ethidium has fluorescent emission at 610 nm in response to 488 nm laser excitation.

2.2. Membrane lipid peroxidation

We used a fluorescent fatty acid conjugate 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid, C11-BODIPY$^{581/591}$ (BODIPY) as a membrane probe for lipid peroxidation [15]. Oxidation of BODIPY by ROS causes an irreversible fluorescence shift from red to green in response to 488 nm laser excitation [15,16]. The assay of BODIPY oxidation is accepted as superior to malondialdehyde analysis as a method of measuring lipid peroxidation in cultured cells [16] and mammalian sperm [15,17]. Subsequent staining of sperm with propidium iodide (PI) allows nonviable cells to be electronically excluded from the analysis and provides an estimate of the proportion of the live population that contains membrane lipid peroxidation [18].

2.3. Mitochondrial transmembrane potential

The percentage of cells with a mitochondrial inner transmembrane potential ($\Delta\Psi_m$) above a value of 80–100 mV can be estimated flow cytometrically using the mitochondrial probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) [19]. This probe is freely permeable to cells and undergoes reversible transformation from a monomer to an aggregate form (J_{agg}) when it binds to membranes having a value for $\Delta\Psi_m > 80$–100 mV [19]. The J_{agg} form is fluorescent at 590 nm in response to 488 nm excitation. We improved a published method of flow cytometric analysis of J_{agg} fluorescence to identify viable sperm with a high $\Delta\Psi_m$ [20], by electronically gating on viable sperm that contained low PI fluorescence [14].

2.4. Specificity of HE and BODIPY oxidation

The ROS generators $FeSO_4$/ascorbate, H_2O_2, and menadione cause early depression of sperm motility without an increase in mortality [14,18]. An experiment was conducted to determine the specificity of $FeSO_4$/ascorbate, H_2O_2, and menadione with respect to HE and BODIPY oxidation. One ejaculate was collected from each of four boars and each sperm sample was divided into four portions for aerobic incubation for 30 and 120 min at 37 °C with four treatments: control (no ROS generator), menadione (30 μM), H_2O_2 (300 μM), and $FeSO_4$/ascorbate (1 and 30 μM, respectively). The specificity of BODIPY and HE oxidation, in response to treatment with three ROS generators for 30 min is shown (Table 1). Basal levels of HE and BODIPY oxidation in the absence of ROS generators was very low, with <1% of viable sperm containing either oxidized HE or BODIPY. The level of basal ROS formation and membrane lipid peroxidation did not change ($P > 0.05$) between 30 and 120 min incubation at 37 °C (data not shown).

Compared with the control, incubation with H_2O_2 or menadione increased ($P < 0.05$) the percentage of sperm with HE oxidation to >80% by 30 min and increased ($P < 0.05$) ethidium fluorescence intensity/cell two to threefold. By contrast, oxidation of HE in the presence of $FeSO_4$/ascorbate did not differ ($P > 0.05$) from that of the control. Compared to the control, $FeSO_4$/ascorbate increased ($P < 0.05$) the incidence of sperm containing BODIPY to 98% and BODIPY fluorescence intensity/cell ninefold at 30 min. Compared to the control, menadione and H_2O_2 had no effect ($P > 0.05$) on the percentage of sperm with BODIPY oxidation or on BODIPY fluorescence intensity). Sperm viability, measured by the number of sperm that excluded PI, averaged 92% and was not affected ($P > 0.05$) by the presence of ROS generators (data not shown).

Table 1
Comparison of the effects of 30 min incubation of boar sperm at 37 °C with H_2O_2, menadione (MEN), and $FeSO_4$-Na ascorbate (FeAc) on hydroethidium (HE) and C_{11}-BODIPY$^{581/591}$ (BODIPY) oxidation[a]

H_2O_2 (μM)	MEN (μM)	FeAc[b] (μM)	Sperm with oxidized HE (%)	Ethidium fluorescence intensity/sperm	Sperm with oxidized BODIPY (%)	BODIPY fluorescence intensity/sperm
0	0	0/0	0.5 ± 0.2a	1.99 ± 0.07a	0.1 ± 0.1a	0.30 ± 0.06a
0	30	0/0	98.4 ± 0.8b	6.44 ± 0.27b	0.3 ± 0.1a	0.28 ± 0.03a
300	0	0/0	83.2 ± 15.6b	4.72 ± 1.04b	1.1 ± 0.3a	0.35 ± 0.04a
0	0	1/30	0.4 ± 0.2a	2.02 ± 0.09a	92.9 ± 5.9b	2.77 ± 0.57b

Within a column, means without a common letter differ ($P < 0.05$).
[a] Values are mean ± S.E.M. for ejaculates from four boars.
[b] FeAc = a combination of $FeSO_4$ and Na ascorbate, expressed as μM/μM.

3. Effects of hypothermic storage

3.1. Effects of freeze-thawing on ROS formation

Semen was collected from eight boars and was frozen in 0.5 mL straws in a programmable freezer and subsequently thawed, as previously described [21]. Fresh and frozen-thawed, Percoll-washed, sperm were incubated at 37 °C for 30 min to determine if basal levels of HE oxidation were increased in viable sperm of frozen-thawed compared to fresh semen, and to compare the effects of a ROS generating system, xanthine (XA) at 1 mM and xanthine oxidase (XO) at 0.1 U/mL, on ROS formation in viable sperm of fresh and frozen-thawed semen. The effects of XA/XO on HE oxidation, percent motile sperm, and viability in fresh and frozen-thawed semen are shown (Table 2). Basal levels of HE oxidation in viable sperm did not differ ($P > 0.05$) between fresh and frozen-thawed semen, with <4% of the sperm containing oxidized HE. The presence of XA/XO increased ($P < 0.05$) incidence of ROS formation in viable sperm of fresh and frozen-thawed sperm with no difference ($P > 0.05$) between the two semen forms. Sperm motility and viability were less ($P < 0.05$) in frozen-thawed compared with fresh sperm. The presence of XA/XO had an additional inhibitory effect on sperm motility ($P < 0.05$) in both fresh and frozen-thawed sperm, but had no additional negative effect on viability in either semen form. We have consistently found that nonviable sperm contained a greater level of ROS, based on increased HE oxidation, supporting the importance of electronic gating in order to obtain an accurate assessment of the viable population.

3.2. Effects of hypothermic liquid storage on membrane lipid peroxidation

This experiment was conducted to compare the effects of hypothermic liquid storage at 17 °C in Beltsville Thawing solution (BTS) on basal and $FeSO_4$/ascorbate-induced membrane lipid peroxidation. Six ejaculates from four boars were extended separately to 30×10^6 sperm/mL in BTS, divided into three portions, and held as follows: at 25 °C for 30 min (Day 0), at 17 °C for 24 h (Day 1), and at 17 °C for 120 h (Day 5). Each portion was Percoll washed and divided into two portions for aerobic incubation at 37 °C for 30 and 120 min, with $FeSO_4$/ascorbate (0/0 or 1/30 μM) as shown (Table 3). The oxidation of BODIPY and viability were measured at 30 min, and motility was measured at 30 and 120 min. The incidence of cells with membrane lipid oxidation and their fluorescence intensity per cell were greater ($P < 0.05$) in $FeSO_4$/ascorbate treated cells than in non-treated cells. However, basal and $FeSO_4$/ascorbate-induced membrane lipid peroxidation did not differ ($P > 0.05$) among days, with 1 and 82%, respectively, of

Table 2
Effects of semen form (fresh and frozen-thawed) and xanthine (XA)/xanthine oxidase (XO) treatment on the oxidation of hydroethidine to ethidium, motility, and viability in boar sperm[a]

Semen form	Incubation	Sperm with ethidium fluorescence (%)	Motility (%)	Viability (%)
Fresh	37 °C	3.5 ± 0.9a	80.0 ± 3.4a	76.7 ± 3.6a
	37 °C, XA/XO	94.4 ± 2.9b	52.1 ± 7.3b	76.2 ± 2.3a
Frozen-thawed	37 °C	2.8 ± 0.3a	20.3 ± 4.4c	32.2 ± 3.1b
	37 °C, XA/XO	87.9 ± 4.4b	7.9 ± 3.0d	32.6 ± 1.4b

Within a column, means without a common letter differ ($P < 0.05$).
[a] Values are mean ± S.E.M. for an ejaculate from each of eight boars.

Table 3
Effects of hypothermic liquid storage in Beltsville Thawing solution on basal and $FeSO_4$-Na ascorbate (FeAc)-induced changes in C11-BODIPY$^{581/591}$ (BODIPY) oxidation in viable boar sperm[a]

Storage time (day)	FeAc[b] (μM)	Sperm with oxidized BODIPY (%)	BODIPY fluorescence intensity/sperm	Motile sperm (%)	Cell viability (%)
0	0/0	1.3 ± 0.3a	0.36 ± 0.04a	79 ± 2.6a	90.1 ± 2.6a
	1/30	75.0 ± 5.5b	1.17 ± 0.12b	58 ± 0.8b	92.6 ± 0.8a
1	0/0	0.8 ±0.2a	0.22 ± 0.02a	72 ± 2.0a	85.7 ± 2.0b
	1/30	81.9 ± 5.5b	1.25 ± 0.14b	30 ± 1.8c	85.2 ± 1.8b
5	0/0	1.0 ± 0.3a	0.25 ± 0.03a	50 ± 1.6b	83.4 ± 1.6b
	1/30	88.6 ± 4.5b	1.58 ± 0.17b	20 ± 2.8c	81.0 ± 2.8b

Within a column, means without a common letter differ ($P < 0.05$).
[a] Values are mean ± S.E.M. for six ejaculates.
[b] FeAc = a combination of $FeSO_4$ and Na ascorbate, expressed as μM/μM.

viable sperm containing oxidized HE. Fluorescence intensity and the number of cells containing oxidized BODIPY were not affected by storage time or the interaction of $FeSO_4$/ascorbate treatment with storage time ($P > 0.05$). The viability on Day 0, 91%, was greater ($P < 0.05$) than the viability on Days 1 and 5 (85.5 and 82%, respectively); viability on each day did not differ ($P > 0.05$) in the presence or absence of $FeSO_4$/ascorbate.

Regarding motility, storage time ($P = 0.001$), $FeSO_4$/ascorbate treatment ($P < 0.0001$), incubation time ($P = 0.004$), and the interaction of $FeSO_4$/ascorbate and incubation time ($P = 0.02$) were sources of variation (data not shown). Compared with no $FeSO_4$/ascorbate, the presence of $FeSO_4$/ascorbate on Day 0 decreased ($P < 0.05$) motility after 120 min of incubation, but not at 30 min. By contrast, on Days 1 and 5, compared with no $FeSO_4$/ascorbate, the presence of $FeSO_4$/ascorbate decreased ($P < 0.05$) motility at both 30 and 120 min (data not shown).

3.3. Effects of freeze-thawing membrane lipid peroxidation

To further confirm the specificity of $FeSO_4$/ascorbate-inducible membrane lipid peroxidation, an experiment was conducted by thawing sperm in the presence of varying concentrations of EDTA serving as an iron chelator. Percoll-washed, frozen-thawed sperm samples from six boars were divided into portions for six treatment combinations of $FeSO_4$/ascorbate and EDTA (μM) (as shown in Table 4), and incubated aerobically for 120 min at 37 °C. The data for oxidation of BODIPY, motility, and viability are shown at 30 min.

Mean viability of thawed sperm in the absence of $FeSO_4$/ascorbate was approximately 64% (Table 4). The presence of $FeSO_4$/ascorbate alone decreased ($P < 0.05$) viability to 48.9%. The presence of EDTA (all concentrations) with $FeSO_4$/ascorbate maintained viability at levels that did not differ ($P > 0.05$) from the absence of $FeSO_4$/ascorbate. The fluorescence of BODIPY in nonviable sperm was not statistically analyzed in this experiment, but the percentage of nonviable sperm with high intensity BODIPY fluorescence was approximately 80%, with a mean fluorescence intensity that was equal to or greater than the mean for viable sperm treated with $FeSO_4$/ascorbate (data not shown).

Motility of thawed sperm in the absence $FeSO_4$/ascorbate (with or without EDTA) was stable at approximately 23% between 30 and 120 min (data not shown), but $FeSO_4$/ascorbate alone decreased ($P < 0.05$) motility at 30 and 120 min compared with no $FeSO_4$/ascorbate (data not shown). The inclusion of 3 or 9 μM EDTA with $FeSO_4$/ascorbate completely blocked the negative effect of $FeSO_4$/ascorbate by maintaining motility at levels similar to sperm incubated in the absence of $FeSO_4$/ascorbate.

4. Impact of ROS formation on sperm energetics

4.1. Effects of menadione

One possible mechanism by which motility is inhibited by ROS formation is through decreased ATP production [22]. This experiment was conducted to use menadione-induced ROS formation as a model to investigate the relationship between motility, $\Delta\Psi_m$, and ATP content (pmol/10^6 cells) by a luciferin-luciferase assay [23]. Percoll-washed sperm from six boars following a 120 min aerobic incubation at 37 °C with 30 μM menadione are shown (Fig. 1). A major portion of the sperm population incubated with menadione was ethidium fluorescent (86.5%) after 30 min (data not shown). Motility and the number of sperm with a

Table 4
Inhibition of $FeSO_4$-Na ascorbate (FeAc)-induced cell death and BODIPY oxidation in viable thawed sperm during 30 min incubation with the metal chelator EDTA[a]

FeAc[b] (μM)	EDTA (μM)	Sperm with oxidized BODIPY (%)	BODIPY fluorescence intensity/sperm	Cell viability (%)
0/0	0	3.4 ± 0.4bc	0.24 ± 0.03bc	65.3 ± 3.3a
0/0	9	1.2 ± 0.2d	0.18 ± 0.02c	63.8 ± 2.8a
1/30	0	75.4 ± 8.3a	1.66 ± 0.38a	48.9 ± 6.5b
1/30	1	71.0 ± 9.5a	1.66 ± 0.39a	61.0 ± 3.4a
1/30	3	5.1 ± 1.2b	0.32 ± 0.06b	63.9 ± 2.8a
1/30	9	2.5 ± 0.6cd	0.22 ± 0.03bc	63.9 ± 2.9a

Within a column, means without a common letter differ ($P < 0.05$).
[a] Values are mean ± S.E.M. for an ejaculate from each of six boars.
[b] FeAc = a combination of $FeSO_4$ and Na ascorbate, expressed as μM/μM.

$\Delta\Psi_m > 80$–100 mV decreased ($P < 0.05$) by >90% between 5 and 60 min. In contrast, ATP content did not decrease until sometime after 60 min.

4.2. Effects of hydrogen peroxide

This experiment tested the direct effects of H_2O_2 on the relationships among motility, $\Delta\Psi_m$, and ATP content. Data are shown (Fig. 2) for Percoll-washed sperm from five boars after aerobic incubation for 120 min at 37 °C with 300 μM H_2O_2. A major portion of the sperm population incubated with H_2O_2 was ethidium fluorescent (70%) after 30 min (data not shown). Motility decreased ($P < 0.05$) by 90% between 5 and 60 min. The decrease in motility was not accompanied by any change in $\Delta\Psi_m$ or ATP content. Viability, estimated by the exclusion of Yo Pro-1, in this experiment and the previous menadione experiment, was maintained at approximately 90% throughout the 120 min incubation period.

5. Discussion

One of the major findings of our studies was that basal ROS formation and membrane lipid peroxidation were very low in viable sperm of fresh and stored boar semen. This is important, because not all cells are endowed with the enzymes that scavenge ROS [7,11]. Theoretically, the first two check points in the cellular ROS defense system are dismutation and then the enzymes catalase and/or glutathione peroxidase to catalyze the reduction of H_2O_2 to water [10,11]. Based

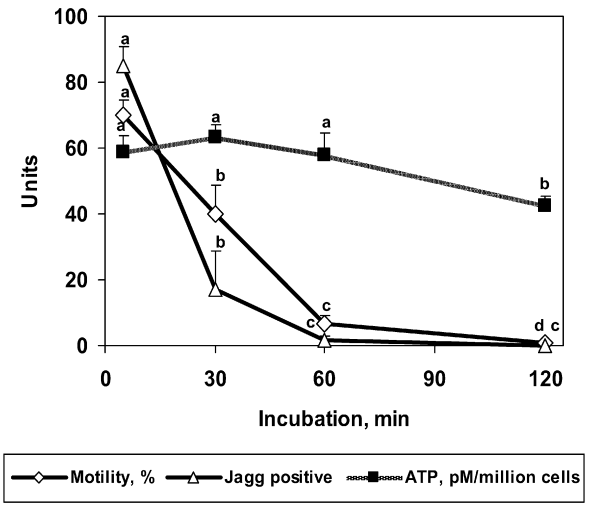

Fig. 1. Effects of menadione treatment on percent motile sperm, percent of viable sperm with a mitochondrial inner membrane potential >80–100 mV (Jagg positive), and ATP (pmol/million cells) during aerobic incubation at 37 °C for 30 min. Values are mean ± S.E.M. Means without a common letter (a–d) differ ($P < 0.05$).

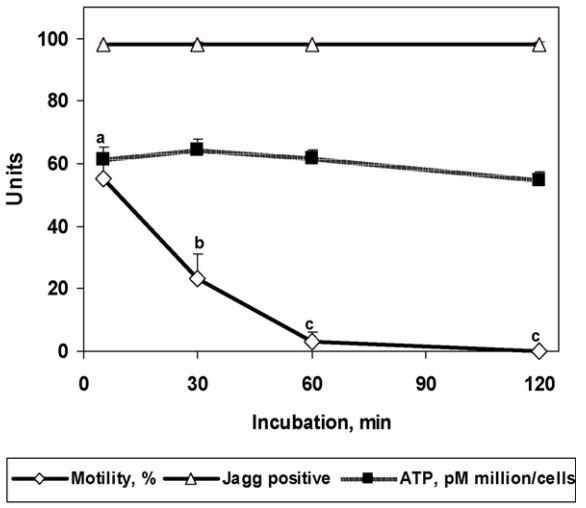

Fig. 2. Effects of H_2O_2 treatment on percent motile sperm, percent of viable sperm with a mitochondrial inner membrane potential >80–100 mV (Jagg positive), and ATP (pmol/million cells) during aerobic incubation at 37 °C for 30 min. Values are mean ± S.E.M. Means without a common letter (a–c) differ ($P < 0.05$).

on the low basal levels of ROS formation and membrane lipid peroxidation, the endogenous ROS defense system in boar sperm is either very efficient or essentially unchallenged, indicating a low level of mitochondrial $^{\bullet}O_2$ formation or sufficient enzymatic activity to neutralize $^{\bullet}O_2$ and H_2O_2.

Surprisingly, we found that the abrupt termination of motility by H_2O_2 was not accompanied by any change in $\Delta\Psi_m$ or sperm ATP content, as reported previously [22]. Perhaps H_2O_2 did not disrupt mitochondrial function, but instead exerted a negative effect in the axoneme to disrupt ATP utilization, or to interfere with the contractile mechanism. Menadione depressed motility and $\Delta\Psi_m$, whereas the decrease in ATP production was small and delayed. In the case of menadione-treated sperm, ATP may have been maintained by glycolysis; in that regard, glycolysis has been proposed as a specialized or sperm-specific source of ATP to maintain motility in mammalian sperm [24–28]. We suggest that the maintenance of ATP after cessation of sperm motility in our studies may be a result of ATP not being required for contractile activity during a period after ROS treatment while the sperm are still alive.

6. Conclusion

The advantage of the flow cytometric analyses of ROS formation and membrane lipid peroxidation is that they can estimate the basal level of ROS in the viable portion of the cell population and can measure a wide range of ROS formed during exposure to oxidative stress; the oxidization of HE by $^{\bullet}O_2$ or H_2O_2 and the oxidation of BODIPY by the action of hydroxyl, alkoxyl, peroxyl radicals, and peroxynitrite. The understanding of sperm energy metabolism, particularly the maintenance of ATP production capacity, is important to optimize liquid semen hypothermic storage and cryopreservation technology.

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